Annex A

BG1Luc ER TA Test Method - Submission from:

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Submission of XDS's LUMI-CELL™ ER High-Throughput System for Screening Estrogen-Like Chemicals for Review by ICCVAM

1.0 Introduction and Rationale for the Proposed Test Method

1.1 Introduction

1.1.1 Describe the historical background for the proposed test method, from original concept to present. This should include the rationale for its development, and overview of prior development and validation activities, and, if applicable, the extent to which the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards.

The association of exposure to endocrine (hormone) disruptor chemicals (EDCs) and adverse health effects in human and wildlife populations has led to worldwide concern. Some of the health effects that have led to this concern include global increases in testicular cancer, regional declines in sperm counts, altered sex ratios in wildlife populations, increases in the incidence of breast cancer and endometriosis, and accelerated puberty in females that are expected to result from exposure to chemicals that adversely affect steroid hormone action (Colborn, vom Saal et al. 1993; Sakr 1993; Adami 1994; Birnbaum 1994; Colborn 1995; LeBlanc 1995; Adami, Bergstrom et al. 1996; Fisch 1996). These observations have focused intense national and international attention on the role of environmental chemicals known as endocrine disruptors (Gray, Kelce et al. 1997; Gray 1998; DeVito, Biegel et al. 1999). The concern over these effects in both human and wildlife populations led to passage of the Food Quality Protection Act and Safe Drinking Water Act by the U.S. Congress (Food Quality Protection Act 1996; Safe Drinking Water Act 1996). These acts mandated the USEPA to investigate the effects of environmental chemicals on the reproductive capacity of both wildlife species and humans. To fulfill this mandate the EPA organized the Endocrine Disruptor Steering and Testing Advisory Committee (EDSTAC); a group of scientists from industry, academia and government; to define a consensus course of action to evaluate potential adverse reproductive effects of a wide range of environmental and industrial chemicals. EDSTAC proposed a tiered testing approach with High-Through Put Pre-Screening (HTPS) reporter gene assays, which would be used to pre-sort chemicals and assist in defining research priorities, provided that these systems are technically feasible and validated. The EDSTAC report was submitted to Congress in August 2000 and resulted in the formation of the Endocrine Disruptor Screening Program (EDSP) within the EPA. The EDSTAC report proposed that EPA pursue the standardization and validation of Tier I and Tier II screening assays for endocrine disruptors. These bioassays would specifically examine the ability of a chemical to act like a hormone (agonist) and/or to block the action of a hormone (antagonist) at the level of gene expression. Some cell lines have been developed in an attempt to fill this role, the majority of these bioassays are not very sensitive or applicable to HTPS protocols. This latter point is extremely important especially considering the tremendous number of chemicals mandated to be tested in addition to many environmentally relevant chemicals and contaminants.

In April 2000 the EPA asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate programs and systems directed toward ER and AR endocrine disruptor *in vitro* binding and transcriptional activation. In response to this request, ICCVAM assembled an expert committee made up of academic, governmental and industry experts who came up with a list of 78 compounds which are recommended for testing for validation of ER and AR endocrine disruptor *in vitro* binding and transcriptional activation test methods (ICCVAM 2002).

Xenobiotic Detection Systems (XDS) in collaboration with Dr. Michael S. Denison (University of California - Davis), has developed a stable recombinant cell line (BG1Luc4E2) that is sensitive and useful in detecting estrogen active chemicals in a HTPS format. XDS is submitting the mechanistic basis for the assay, protocols that are reliable in estimating estrogenic activity of chemicals and mixtures, and data on chemicals we have evaluated for estrogen activity. XDS is submitting this information for review by ICCVAM for its potential as a validated regulatory method in response to the Federal Register Notice (Vol. 66, No. 57/Friday, March 23, 2001) as a HTPS method for estrogen active compounds. We are in the process of developing other recombinant cell based assays that would be useful in fulfilling the federally mandated need for analytical systems that can identify chemicals with endocrine disruptor activity.

Receptor-Dependent Mechanism of Action of Estrogen-Steroid Hormones and Effects of EDCs.

The molecular mechanism of action of estrogen-steroid hormones is based on their ability to bind to and activate specific nuclear receptor proteins in responsive cells (Carson-Jurica, Schrader et al. 1990; Beato, Herrlich et al. 1995). A drawing depicting the molecular mechanism of estrogen activation of gene expression and biochemical events that occur following exposure of cells to estrogen or estrogenic chemicals is shown in figure 1. Estrogen or chemicals that act as agonists for the estrogen receptor bind to the receptor and then the receptor dimerizes to the ligand-activated form of the receptor that can bind to DNA sequences (Estrogen-response-elements, EREs), that are upstream of estrogen responsive genes. Binding of the ligand activated receptor complex results in initiation of transcription of the down stream-associated genes under control of the EREs. Environmental EDCs can adversely affect hormone action by exerting an effect on one or more steps in these ligand- and receptor-dependent signal transduction pathways. Chemicals can bind to these receptors and directly activate the receptor or inhibit (antagonize) the binding and activation of the receptor by its endogenous ligand (estrogen).

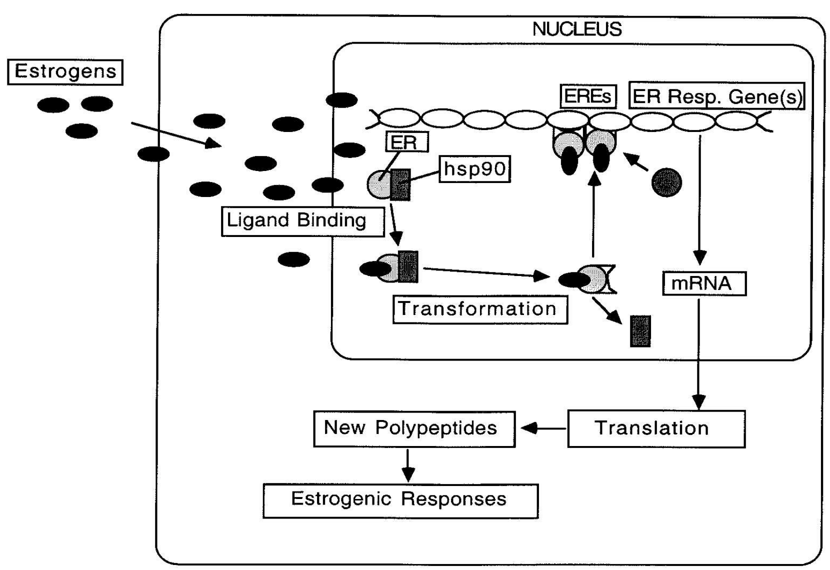

Figure 1. Molecular mechanism of estrogen hormone action. See text for details.

Describe the purpose, including the mechanistic basis, of the proposed test method.

The test method was developed by producing a recombinant cell line that contains a reporter construct that expresses luciferase activity in response to exposure of the cells to estrogen or estrogen-like compounds. Shown in figure 2 is the plasmid pGudLuc7.ERE used to produce a recombinant cell line. This plasmid contains 4 copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene. BG1 a human ovarian carcinoma that expresses the estrogen receptor was transfected with the reporter gene construct and stable transfectants selected by growth in MEM containing 0.4 mg/ml geneticin (G418) until colonies inducible for luciferase activity were cloned (Rogers and Denison 2000). A stably transfected cell line designated BG1Luc4E2 was cloned from this procedure and expresses luciferase activity in response to estrogen and estrogen-like chemicals. The cell line BG1Luc4E2 has demonstrated stable induction of luciferase activity in response to exposure of the cells to estrogen for over 5 years. The conditions to grow these cells and measure the estrogen-inducible expression of luciferase activity in a high-throughput screen format are defined in this document.

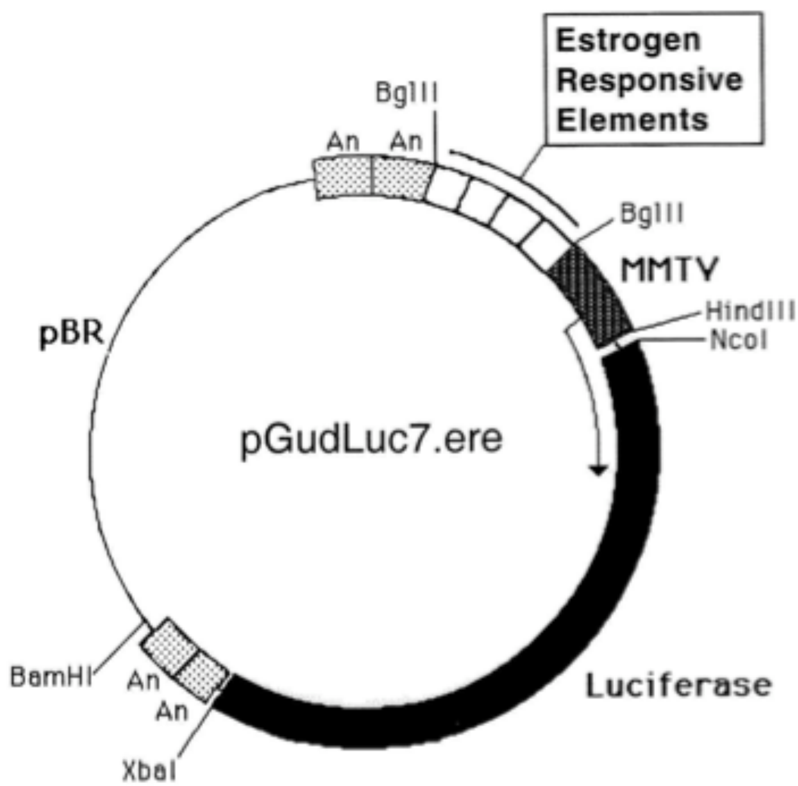

Figure 2. Plasmid pGudLuc7.ERE used to produce recombinant cell line BG1Luc4E2.

When possible, describe what is known about the similarities and differences of modes and mechanisms of action in the test system as compared to the species of interest (e.g., humans for human health-related toxicity testing).

There are other mechanisms by which chemicals may demonstrate endocrine disruptor activity in addition to directly competing for ligand binding to the estrogen receptor. EDCs can stimulate metabolic degradation or synthesis of the endogenous hormone ligand or receptor itself and/or indirectly activate or inhibit activation of the receptor by affecting receptor phosphorylation (*i.e.* by stimulating or repressing protein kinases or phosphatases known to be important in receptor function) (Spink, Lincoln et al. 1990; Spink, Eugster et al. 1992; Weigel and Zhang 1998). Additional targets for EDCs include chemical-dependent alterations in the expression of a given receptor(s) and/or the level, or function, of a critically important nuclear receptor coactivator or corepressor necessary for receptor functionality. Thus, it is possible for xenobiotic chemicals to alter normal endocrine homeostasis and hormone action both directly and indirectly by a variety of different mechanisms in different cell types. However, the primary mechanism for chemicals to act as endocrine disruptors is through acting as an agonist or an antagonist of the hormone at the receptor level altering gene expression. Irrespective of the above actions of an EDC, effects of all of these targets will result in a change in ER-dependent gene expression. Thus, the cell line BG1Luc4E2 is a useful tool for HTPS analysis of chemicals for potential activity as estrogenic agonists or antagonists of gene expression. XDS has termed the bioassay using BG1Luc4E2 as the LUMI-CELL™ ER test.

1.1.2 Summarize and provide the results of any peer review conducted with the test summarize and ongoing planned reviews.

There has been one peer reviewed published paper on the production of the BG1Luc4E2 (Denison, Phelan et al. 1998), one paper on the LUMI-CELL™ ER test (Gordon, Chu et al. 2003), and one paper comparing the XDS transcriptional assay with immature mouse uterotrophic responses in assessing the estrogenic activity of phytochemicals (see the attached papers in Appendix H) (Jefferson, Padilla-Banks et al. 2002). Briefly, the LUMI-CELL™ ER bioassay (termed the ER Transcriptional assay in the Jefferson 2002 paper) demonstrated estrogen agonist activity for all the compounds tested except Taxifolin. The compounds demonstrating estrogen agonist activity were (17b-estradiol, DES, Zeralanol, Zeralenone, Coumesterol, Genistein, Biochanin A, Daidzein, and Naringenin. Other in vivo bioassay systems that were used to assess estrogen agonist activity were the Uterotrophic assay (measurement of uterine wet weight increase in immature mice), increase in uterine epithelial cell height, uterine Gland number increases, and induction of estrogen responsive protein lactoferin (LF assay). The Uterotrophic assay was able to detect estrogenic activity in 7 of the 10 compounds. The uterine cell height assay was able to detect estrogenic activity in 9 of the 10 compounds. The uterine Gland number assay was able to detect estrogenic activity in all 10 of the compounds tested, making it very consistent with the LUMI-CELL™ ER bioassay. Immunohistochemical analysis of lactorferin (LF) induction was able to detect estrogenic activity in 7 of the 10 compounds detected by the LUMI-CELL™ ER bioassay (Jefferson, Padilla-Banks et al. 2002). This data demonstrates the unique sensitivity of the LUMI-CELL™ ER bioassay in evaluating estrogenic activity of these phytoestrogens and agreement with an in vivo model to evaluate estrogen agonists. Taxifolin was either a non-active or very weakly active in all of the above assays.

XDS has been in contact with members of NICEATM (Dr. William Stokes and Dr. Raymond Tice) to keep them informed on progress we have made in development of the LUMI-CELL™ ER test. Updates of the development included a site visit to the XDS laboratories and a number of meetings to review data and evaluate needs of an estrogenic agonist and antagonist assay of chemicals for potential endocrine disrupting activity.

1.1.3 Clearly indicate any confidential information associated with the test method; however inclusion of confidential information is discouraged.

Confidential information is included in this submission. The software that has been developed for automated analysis and the use of Hill equation modeling of receptor mediated gene expression is novel and under copyright submission. Information on these data analysis systems is included in Figure 5 of the submission. Raw data will also be provided on CD in Excel file format.

1.2 Regulatory rationale and applicability

1.2.1 Describe the current regulatory testing requirement(s) for which the proposed test method is applicable.

The proposed method is suggested as a primary HTPS assay for chemicals that display estrogenic or anti-estrogenic activity. A major portion of the EDSTAC report to congress suggested that reporter gene technology may be useful for priority setting in screening chemicals that are potential endocrine disruptors [Endocrine Disruptor Screening Program, August 2000]. It was also suggested that in vitro screening would provide relative potency and dose response data that could be used to set doses in animal tests that follow screening. This could have a significant impact on reducing the number of animals used in animal testing protocols in Tier 1 and Tier 2 tests for endocrine disruptors. ICCVAM's expert panel has recommended 78 compounds to be tested during the validation process of ER and AR endocrine disruptor *in vitro* binding and transcriptional activation methods. The pharmaceutical industry has used HTPS reporter gene technology for identifying chemicals with properties that may be useful as drug candidates. The EPA awarded a contract to evaluate reporter gene technology as a screen for endocrine active chemicals. EPA concluded from this preliminary evaluation of reporter gene technology that the technology was not sufficiently sensitive or robust for identifying EDCs for regulatory purposes [Endocrine Disruptor Screening Program, August 2000]. XDS has developed extremely sensitive reporter gene systems to analyze for trace contamination of chemicals in the environment and should be useful for evaluating EDCs. This submission of data contains information on the HTPS format use of the LUMI-CELL™ ER bioassay. It is necessary for ICCVAM and the regulatory agencies to evaluate the robustness and sensitivity of reporter gene technology to be useful for regulatory purposes.

1.2.2 Describe the intended regulatory use(s) (e.g., screen, substitute, replacement, or adjunct) of the proposed test method and how it will be used to substitute, replace or complement any existing regulatory requirement(s).

The method is intended as a screen to identify chemicals that may possess estrogenic activity and for priority setting in the tiered approach for identifying endocrine disruptor active chemicals. The EDSTAC report suggests a tiered approach for evaluating chemicals for endocrine disruptor activity with HTPS of estrogen, androgen and thyroid hormones to be used in priority setting if these systems can be validated. The LUMI-CELL™ ER reporter gene system should be one of these systems that provide data for the evaluation of chemicals for estrogenic activity. The EPA has suggested that Quantitative Structure-Activity Relationships (QSAR's) can be used in their priority setting for evaluating chemicals for endocrine disruptor activity. Data from our estrogen reporter system can be used as information to populate the database of QSAR's for the evaluation of the estrogenic activity of chemicals. Results of tests with the LUMI-CELL™ ER reporter gene system can also be used to compare results of other Tier 1 and 2 systems that are being evaluated such as the Uterotrophic Screen, The Hershberger Screen, The Rodent Pubertal Female Screen, The Rodent Pubertal Male Screen, Fish Reproduction Screen, The Frog Metamorphosis Screen, Estrogen and Androgen Receptor Reporter Gene Screens and Other In Vitro Screens, Mysid Shrimp (Invertebrate) Reproduction Test, and Mammalian 2-Generation Reproduction

Test [Endocrine Disruptor Screening Program, August 2000]. Studies have been initiated to evaluate the LUMI-CELL™ ER in vitro system for its efficacy in identifying estrogen agonists versus the Uterotrophic Screen and other in vivo endpoints of estrogen activity (Jefferson, Padilla-Banks et al. 2002). The Uterotrophic screen has been proposed as one of the primary assays to identify estrogenic chemicals.

1.2.3 Where applicable, discuss the similarities and differences in the endpoint measured in the proposed test method and the currently used *in vivo* reference test method and, if appropriate, between the proposed test method and a comparable validated test method with established performance standards.

The LUMI-CELL™ ER reporter gene system can be performed much more rapidly and economically than the Tier 1 and Tier 2 systems being evaluated by the EPA listed above. The EPA has awarded a contract to the Battelle Corporation to validate these in vivo methods, but no studies have been published or validation parameters reported to our knowledge. There is a good correlation between the LUMI-CELL™ ER assay and the Uterotrophic assay (Thigpen, Locklear et al. 2001; Jefferson, Padilla-Banks et al. 2002). XDS has generated some data comparing the responsiveness of the LUMI-CELL™ ER reporter gene system to the mouse Uterotrophic Screen for evaluating the estrogenic activity of various feed substances. These preliminary analyses are single blinded studies being conducted with Dr. Julius Thigpen at the National Institutes of Environmental Health Sciences. Preliminary results appear promising that the LUMI-CELL™ ER reporter gene system is predictive for estrogen active contaminants in feeds that cause a response in the mouse Uterotrophic Screen. Comparison of the LUMI-CELL™ ER reporter gene system should be undertaken with the other tests EPA is evaluating (listed above in section **1.2.1**), particularly since these tests are much more complex, many require the use of animals, and are much more costly.

There is also a paper discussed in section 1.1.2 comparing the XDS transcriptional assay, with immature mouse uterotrophic responses in assessing the estrogenic activity of phytochemicals (see the attached papers in Appendix H) (Jefferson, Padilla-Banks et al. 2002). Briefly, the LUMI-CELL™ ER bioassay (termed the ER Transcriptional assay in the Jefferson 2002 paper) demonstrated estrogen agonist activity for all the compounds tested except Taxifolin. The compounds demonstrating estrogen agonist activity were (17b-estradiol, DES, Zeralanol, Zeralenone, Coumesterol, Genistein, Biochanin A, Daidzein, and Naringenin. Other in vivo bioassay systems that were used to assess estrogen agonist activity were the Uterotrophic assay (measurement of uterine wet weight increase in immature mice), increase in uterine epithelial cell heightuterine Gland number increases, and induction of estrogen responsive protein lactoferin (LF assay). The Uterotrophic assay was able to detect estrogenic activity in 7 of the 10 compounds. The uterine cell height assay was able to detect estrogenic activity in 9 of the 10 compounds. The uterine Gland number assay was able to detect estrogenic activity in all 10 of the compounds tested, making it very consistent with the LUMI-CELL™ ER bioassay. Immunohistochemical analysis of lactorferin (LF) induction was able to detect estrogenic activity in 7 of the 10 compounds detected by the LUMI-CELL™ ER bioassay (Jefferson, Padilla-Banks et al. 2002). This data demonstrates the unique sensitivity of the LUMI-CELL™ ER bioassay in evaluating estrogenic activity of these phytoestrogens and agreement with an in vivo model to evaluate estrogen agonists. Taxifolin was either a non-active or very weakly active in all of the above assays.

1.2.4 Describe how the method fits into the overall strategy of hazard or safety assessment. If a component of a tiered assessment process, indicate the weight that should be applied relative to other measures.

The LUMI-CELL™ ER reporter gene system is a mechanistically based ER-receptor bioassay system to identify chemicals that possess estrogen activity. The test system allows for specificity to be evaluated, since luciferase activity is not induced unless the estrogen receptor has been activated. A number of assays have been proposed using estrogen driven growth of cells as a screen for estrogen activity (Soto, Sonnenschein et al. 1995; Soto, Michaelson et al. 1998). However, effects of chemicals on a vast array of biochemical pathways can affect cell growth independent of the estrogen response. The specificity and sensitivity of dose response data that compares the relative estrogen activity of chemicals is one of the assets of the LUMI-CELL™ ER reporter gene system.

Describe the intended range of materials amenable to the test and/or the limits of the proposed test method according to chemical class or physico-chemical factors.

The range of materials that can be tested is limited only by their solubility in Dimethyl Sulfoxide (DMSO) or other solvents compatible with the cell line that do not produce toxicity. The solvent DMSO can solubilize a wide range of compounds having both hydrophobic and hydrophilic characteristics. The one characteristic that limits the test system is that the chemical, solvent, or extract being tested should not be toxic to the cell system. Cell toxicity would result in a potential false negative response for estrogenic activity of the test chemical. However, the large dynamic range for induction of luciferase activity in the LUMI-CELL™ ER reporter gene system allows for dilution of the chemical or extract to a concentration at which toxicity is minimal and estrogenic activity of the compound may still be evaluated.

1.3 Scientific basis for the proposed test method.

1.3.1 Describe the purpose and mechanistic basis of the proposed test method.

The primary purpose of the LUMI-CELL™ ER bioassay is to screen chemicals for potential estrogenic activity. Eventually expanding to test feed, food and consumables for contamination for potential estrogenic activity. The mechanistic basis for this test method was described in section 1.1.1.

1.3.2 Describe what is known and not known about the similarities and differences of modes and mechanisms of action in the proposed test method as compared to the species of interest (e.g., humans for human health related toxicity testing).

The proposed test method uses human ovarian carcinoma cell line BG-1, which has an endogenous estrogen receptor. The plasmid construct described in section **1.1.1** has 4 copies of the vitelogenin estrogen receptor response element in series placed in front of the reporter gene. The mechanism is very similar in humans for activation of the estrogen receptor and then regulation of gene expression on a wide variety of genes under control of the estrogen receptor.

1.3.3 Describe the intended range of substances amenable to the proposed test method and/or the limits of the proposed test method according to chemical class or physicochemical standards.

As described in section **1.2.4**, the range of materials that can be tested is limited only by their solubility in Dimethyl Sulfoxide (DMSO) or other solvents compatible with the cell line that do not produce toxicity. The solvent DMSO can solubilize a wide range of compounds having both hydrophobic and hydrophilic characteristics. The one characteristic that limits the test system is that the chemical, solvent, or extract being tested should not be toxic to the cell system. Cell toxicity would result in a potential false negative response for estrogenic activity of the test chemical. However, the large dynamic range for induction of luciferase activity in the LUMI-CELL™ ER reporter gene system allows for dilution of the chemical or extract to a concentration at which toxicity is minimal and estrogenic activity of the compound may still be evaluated.

2.0 Test Method Protocol Components

2.1 Provide and overview of how the proposed test method is conducted. If appropriate, this would include the extent to which the protocol for the proposed test method adheres to established performance standards.

See Appendix A – Detailed description of Performance of the LUMI-CELL™ ER bioassay.

Brief flow chart explanation:

Thaw cells form liquid nitrogen

⬇

Grow in RPMI 1640

⬇

Grow in DMEM for 4 days

⬇

Plate cells in 96 well plates

⬇

Dose Plates with all standards and compounds of interest to be tested.

⬇

Incubate for 20-24 hours and read 96 well plates in Luminometer.

2.2 Provide a detailed description and rationale, if appropriate, for the following aspects of the proposed test method:

2.2.1 Materials, equipment, and supplies needed:

Equipment:

Equipment Item name	Fisher Scientific[1] Cat. #	Price, US $
Class II biological safety hood and stand	16-108-99	$7,250.00
Cell culture incubator, with CO_2 and temp. control	11-689-4	$4,197.00
Centrifuge, low speed, tabletop	04-978-50	$915.00
with swinging bucket rotor	05-103B	$430.00
Drummond diaphragm pipettor	13-681-15	$180.00
Microscope, inverted	12-561-INV	$4,400.00
Microscope	12-561-3M	$750.00
Hemocytometer, cell counter	02-671-5	$105.00
Hand tally counter	07-905-6	$27.72
Micropipettor, 0.5-10 µL range	21-377-97	$199.00
Micropipettor, 40-200 µL range	21-377-99	$199.00
Refrigerator/freezer	13-986-106A	$1,715.00
Vortex – mixer	12-814	$207.00
Vacuum pump with liquid trap (side arm erylenmeyer flask	01-092-29	$316.00
Multipipettor, repeating - syringe type	21-380-8	$390.00
Centrifuge concentrator with vacuum pump with cold trap	16-315-45	$5,595.00
Shaker for 96 well plates	14-271-9	$790.00
Liquid Nitrogen dewar	11-675-92	$1,154.00
or -70 celcius freezer	13-989-187	$7,350.00
Luminometer Berthold		$19,920.00
and dedicated computer		$1,679.00
Combustion test kit, CO_2 monitoring	10-884-1	$341.25
13mm test tube racks	14-809-22	$14.36
13mm test tube racks for dosing	14-810-54A	$16.99
16 mm test tube racks	14-809-24	$14.36
50 ml test tube racks	14-809-28	$15.71
sonicating water bath	15-335-30	$505.50

The recombinant cell line BG1Luc4E2, licensing arrangements can be made with XDS for use of this cell line.

Supplies:

Cell Culture

9" Pasteur pipettes
pipette bulbs, 2 ml capacity, pack of 72
15 ml plastic centrifuge tubes, sterile
50 ml plastic centri. Tubes
13/100 test tubes
Phosphate buffered saline
RPMI and DMEM medium
Trypsin
pen/strep solution
Fetal serum
RPMI Fetal medium
Lysis Solution
Substrate Solution
75 cm2 tissue culture flasks
96 well plates
Backing Tape
70 % ethanol, for cleaning and as coolant for cold trap
latex gloves
p200 pipette tips, sterile
2 ml sterile pipettes-plastic, case of 500
10 ml sterile pipettes, plastic, case of 200
1.0 ml multipipettor syringes, case of 100
10.0 ml multipipettor syringes, case of 100
sodium hydroxide
DMSO
Minimal Essential Medium
Estrogen stripped fetal calf serum

2.2.2 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting the test, if applicable.

The dose selection for 17b-estradiol standard is based upon the responsiveness of our genetically engineered BG1Luc4E2 cells to estrogen. The cells are extremely sensitive to estrogen and estrogen-like chemicals demonstrating a significant agonistic response to as little as 0.4 pg of 17b-estradiol. The BG1Luc4E2 cells respond with a dose dependent induction of luciferase activity up to a maximal concentration of 40 pg of 17b-estradiol. A Tamoxifen / 17b-estradiol mixture was used in the antagonistic response test and demonstrates significant responses to Tamoxifen in the range from 1.95 x 10^3 pg – 2.0 x 10^6 pg (with a constant 10 pg 17b-estradiol concentration).

A screening testing for both agonistic and antagonistic estrogenic activity of a chemical is performed by initially performing a dose range finding experiment with the chemical. For the agonist response, ten milligrams of a pure chemical for testing of estrogenic activity is weighed out into glass vial and dissolved in one-milliliter of DMSO. A 10 fold dilution series of the chemical is then produced by adding 10 microliters of the test compound to 90 microliters of DMSO in a 13 mm glass tube and repeating this procedure for six dilutions creating a dilution series of 1 mg/ml down to 1 ng/ ml. Four microliters of these solutions is then added to 400 microliters of media (final concentrations of 10 micrograms/ml down to 10 picrograms/ml) and applied to the BG1Luc4E2 cells to evaluate induction of luciferase activity. Using this screening format, 8 compounds can be evaluated per plate of BG1Luc4E2 cells. If a test chemical is positive for induction of luciferase activity a second experiment using a two fold dilution series at the concentrations that are active is performed. An example two-fold analysis of the activity of diethlstilbesterol was included in the example analysis provided from 50 pg/ml down to a concentration of 1.56 pg/ml.

The dosing method for the antagonist response was conducted in much the same way as the agonist response with some small changes. Ten milligrams of a pure chemical for testing of antagonist estrogenic activity is weighed out into glass vial and dissolved in one-milliliter of DMSO. A 10 fold dilution series is of the chemical was again produced by adding 10 microliters of the test compound to 90 microliters of DMSO in a 13 mm glass tube and repeating this procedure for six dilutions creating a dilution series of 1 mg/ml down to 1 ng/ml. Four microliters of these solutions along with 10 pg/ml 17b-estradiol is then added to 400 microliters of media (final concentrations of 5 μg/ml down to 5 pg/ml of the compound and 10 pg/ml 17b-estradiol in each tube) and applied to the BG1Luc4E2 cells to evaluate the reduction in induction of luciferase activity. Using this screening format, 8 compounds can also be evaluated for antagonistic activity per plate of BG1Luc4E2 cells. If a test chemical is positive for reduction of luciferase activity, a second experiment using a two fold dilution series at the concentrations that are active is performed. Tamoxifen was used as the standard for the antagonistic response. Tamoxifen gave responses in the range from 1.95 x 10^3 pg – 2.0 x 10^6 pg. IICI 182,780 was not used as the standard for antagonistic response due to cost and not being readily available (i.e. only 100 mg per customer per year).

2.2.3 Endpoint(s) measured;

The endpoint measured is the induction of luciferase activity in a human ovarian carcinoma, BG-1 that has been genetically engineered with a reporter gene construct that expresses the enzyme luciferase in response to exposure of the BG1Luc4E2 cell line to estrogen or estrogen-like chemicals. The light produced can be easily quantified with a luminometer and comparison with a standard of b-estradiol induction of luciferase activity.

2.2.4 Duration of exposure;

The duration of exposure to b-estradiol to induce maximal expression of the luciferase reporter gene in our BG1Luc4E2 bioassay is 24 hours. A significant induction of estrogen dependent expression of luciferase activity can be measured as early as two hours after exposure of the cells with half maximal induction occurring at eight hours following exposure of the BG1Luc4E2 cells (Rogers and Denison 2000).

2.2.5 Known limits of use;

The only known limits of use of the LUMI-CELL™ ER bioassay for measuring estrogen dependent induction of luciferase activity is if the chemical or environmental extract is toxic to the cellular system. Toxicity could potentially inhibit induction of estrogen-dependent induction of luciferase activity. However, overt toxicity is assessed in the system by visual observation of the cells before measurement of luciferase induction, and through a cell viability test. The cell viability test consists of mixing the highest concentration of a compound which demonstrates a negative response, yet has normal cell morphology, with the EC 50 concentration of 17b-estradiol and 1/10th the EC 50 concentration of 17b-estradiol. One or both of these should give a positive response if the compound is negative, demonstrating the cells are still viable. If there is no response the concentration dosed has proven to be toxic to the cells (see results in **Appendix D,** for a data summary; **Appendix G,** for QC summary charts; and Excel file "**Appendix – D Plate-to-Plate Agonist Raw Data**" – cell viability tab, for raw data for Appendix D). However, the sensitivity and large dynamic range of the LUMI-CELL™ ER bioassay system allows for dilution of the sample test compound to limit toxicity and yet estimate potential induction of estrogen-dependent luciferase expression.

2.2.6 Nature of the response assessed;

The response that is measured is the enzymatic activity of luciferase that is induced in our genetically engineered cells (BG1Luc4E2) that express this enzyme in response to exposure to estrogen and estrogen-like chemicals. The enzyme activity is assessed by the production of light in a luminometer following addition of enzyme reagents.

2.2.7 Appropriate vehicle, positive, and negative controls and the basis for their selection;

The vehicle used for application of chemicals is DMSO. The response from the vehicle is the negative control for chemicals and solvent for extraction of environmental samples is the vehicle in testing environmental extracts. The positive controls include an eleven point 17b-estradiol dose response curve, which is the hormone ligand for the estrogen receptor (**Appendix C**, for example of 17b-estradiol curve; **Appendix D**, for data summary; and Excel file "**Appendix – D Plate-to-Plate Agonist Raw Data**" – beta curve tab, for raw data), 4 DMSO controls and one no DMSO (just media), as well as 3 to 8 positive response QCs (**Appendix G**, for QC summary charts). The following compounds are used as QCs for the LUMI-CELL™ ER bioassay and were selected based on historical data provided by ICCVAM and their consistent response in this assay: diethlstilbesterol (DES) (1.23×10^{-5} μg/ml); Bisphenol A (7.81×10^{-2} μg/ml); Estrone (7.81×10^{-2} μg/ml); Ethelene Estradiol (6.25×10^{-2} μg/ml); Feneramol (12.5 μg/ml); Kaemoferol (7.81×10^{-2} μg/ml); Methoxychlor (1.56 μg/ml); Norethredrel (3.13×10^{-5} μg/ml). DES, Bisphenol A, and Estrone are used as the standard QCs for the plates, however the others can be added as needed for specific assays. The QC performance charts are provided here in **Appendix G**.

2.2.8 Acceptable range of vehicle, positive and negative control responses and the basis for the acceptable ranges;

The acceptable range for the vehicle (DMSO) response is less than 20% of the maximal induction of the media used in the dosing portion. QC control charts have been developed for all of the QC compounds mentioned in the above section (section **2.2.7**, See also **Appendix G**). A limit of 2 standard of deviations from the mean has been established to evaluate the acceptability of the QC data and the plate data. Also a minimum induction of 3 has been established for the evaluation of the 17b-estradiol dose response curve.

2.2.9 Nature of the data to be collected and the methods used for data collection;

The data collected are measurements of the light induction produced by the luciferase enzyme and are measured as relative light units detected by a luminometer. The data are stored as electronic files in a computer system that is backed up daily. They are secured in the laboratory and follow methods described in EPA method 2185: Good Automated Laboratory Practices.

2.2.10 Type of media in which data are stored;

The data are stored electronically in a Windows NT network. The network hard disk is backed up every 24 hours on a Compaq workstation. Data printouts are also kept in laboratory notebooks.

2.2.11 Measures of variability;

In the screening mode of the assay replicate analysis are not performed, however the use of a varying doses of compound allows an estimate if the response demonstrates a trend. However, in confirmation assays, triplicate analysis can be performed on both "plate to plate" variability and "well-to-well" variability, and statistical model testing is performed on this data. Testing of compounds was done in the confirmation assay mode and the data is available in **Appendix D**, for Plate-to-Plate Agonist data summary; Excel file "**Appendix – D Plate-to-Plate Agonist Raw Data**", for raw data of Appendix D summary; **Appendix E**, for Plate –to-Plate Antagonist data summary; Excel file "**Appendix – E Plate-to-Plate Antagonist Raw Data**", for Plate-to-Plate Antagonist raw data, **Appendix F**, for Well-to-Well Agonist data summary, and Excel file "17b-estradiol", for Appendix F raw data.

2.2.12 Statistical or non-statistical method(s) used to analyze the resulting data (including methods to analyze for a dose-response relationship). Justify and described the method(s) employed;

The data that is generated from the 17b-estradiol standard is modeled using a four parameter Hill equation. The Hill equation is a mathematical model that generates the best fit for receptor mediated induction of gene expression (Kohn, Lucier et al. 1993; Kohn, Sewall et al. 1996; Kohn, Walker et al. 2001).

2.2.13 Decision criteria or the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate;

There have been three initial criteria adopted for assigning a positive designation for a chemical in the LUMI-CELL™ ER estrogen screen. The first criteria is that the chemical induces luciferase activity that is greater than 3 times the standard deviation of the DMSO blank at an applied concentration of 10 micrograms/ml (see **Appendix D**, data summary). The second more restrictive criteria are that the chemical induces LUMI-CELL™ ER bioassay system at both 10 and 1 microgram/ml (see **Appendix D**, data summary). The third criteria is that the chemical induces luciferase activity at a number of concentrations in a two-fold dilution re-analysis demonstrating dose-dependent induction of luciferase and a relative response to 17b-estradiol can be assigned (see **Appendix D**, data summary). A negative designation for activity in the LUMI-CELL™ ER bioassay estrogen screen is assigned when no induction of luciferase activity is detected at any concentration over 3 times the standard deviation of the DMSO blank (see **Appendix D**, data summary).

2.2.14 Information that will be included in the test report.

Information in test reports include the standard curve generated by a two-fold dilution series of the positive control chemical 17b-estradiol (**Appendix C**, and example of the 17b-estradiol curve; **Appendix D**, data summary; and Excel file "**Appendix – D Plate-to-Plate Agonist Raw Data**" – beta curve tab, for raw data), background determinations of solvent carrier (DMSO), QC (**Appendix G**) and Cell Viability performance charts, "Plate-to-Plate" and "Well-to-Well" variability, antagonist response (**Appendix D**, Agonist Plate-to-Plate data summary; Excel file "**Appendix – D Plate-to-Plate Agonist Raw Data**", for Appendix D raw data; **Appendix E**, Antagonist data summary; Excel file, for Appendix E raw data; **Appendix F**, for Agonist Well-to-Well data summary; and Excel file "**Appendix – F Well-to-Well Agonist Raw Data**", for Appendix F raw data), modeling of the 17b-estradiol response using a four parameter Hill equation, and response of compound range finding at six different 10 fold dilutions from 10 micrograms/ml down to 10 picrograms/ml in our LUMI-CELL™ ER bioassay.

2.3 Explain the basis for selection of the test method system. If an animal model is being used, this should include the rationale for selecting the species, strain or stock, sex, acceptable age range, diet, and other applicable parameters.

The LUMI-CELL™ ER bioassay is an *in vitro* system using a genetically engineered cell line and is a mechanistically based ER-receptor bioassay system to identify chemicals that possess estrogen activity. This test method should greatly reduce, refine and in some cases replace animal use in discovery of estrogenic endocrine potency.

2.4 If the test method employs propriety components, describe what procedures are used to ensure their integrity (in terms of reliability and accuracy) from "lot-to-lot" and over time. Also describe procedures that are used to verify the integrity of the proprietary components.

The integrity of the proprietary LUMI-CELL™ ER bioassay is maintained by several means. The First is the standard 17b-estradiol dose response curve. The cells must respond in a standard sigmoidal shaped curve with an induction of grater than three. Also, a minimum of 8 additional positive and negative QCs, are used in each plate to evaluate the cells integrity. Three are positive QCs (usually DES, Bisphenol A, and Estrone (see **Appendix G** for QC performance charts)) and 5 negative controls (4 DMSO and 1 no DMSO (i.e. just media)). These QCs are checked against established QC charts described in section **2.2.7** and **Appendix G**. The BG1Luc4E2 cell line is also stored in liquid nitrogen, which preserves the integrity of the cell system.

2.5 Describe the basis for the number of replicate and repeat experiments; provide the rationale if studies are not replicated or repeated.

In this study triplicate analysis was preformed on all samples. Samples were analyzed in a "Plate-to-Plate" format where each analysis was done on a completely different experimental setup (**Appendix D**, Agonist Plate-to-Plate data summary) as well as on a "Well-to-Well" format, where 3 of the same samples was analyzed three times on the same plate from the same experimental setup (**Appendix F**, Agonist Well-to-Well data summary). But as described in section 2.2.11, in the screening mode of the assay replicate analysis are not performed, however the use of a varying doses of compound allows an estimate if the response demonstrates a trend. However, in confirmation assays, triplicate analysis can be performed on both "Plate-to-Plate" variability and "Well-to-Well" variability, and statistical model testing may be performed on this data.

2.6 Discuss the basis for any modifications to the proposed that were made based on results from validation studies.

Validation studies are currently in progress with the LUMI-CELL™ ER bioassay analysis system. Modification of protocols will be advanced after sufficient testing demonstrates that modifications improve the systems.

2.7 If applicable, discuss any differences between the protocol for the proposed test method and that for a comparable validated test method with established performance standards.

XDS Inc. is not aware of any validated test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix H**).

2.8 Explain the basis for the decision criteria established for the test.

The decision criteria that we have initially established to identify estrogen agonists by the LUMI-CELL™ ER reporter gene system are explained in section **2.1.14** above. These criteria allow for some indication that a dose dependent induction of luciferase activity is occurring in the system. This should be one of the criteria for establishing whether a chemical is an endocrine active compound. A second criterion should be that a significant response over background is generated. We set a cut-off point of 3 fold increase over the standard deviation of the background as potential noise in the system. This level for discriminating noise or background is accepted for other EPA validated analytical systems such as Method 8290 for analysis of dioxin chemical contamination. The scientific community has not identified criteria for classifying chemicals for endocrine disruptor activity with any certainty at this time. XDS is submitting this system as a mechanistically based ER-receptor bioassay system as a HTPS for Tier 1 priority setting in further evaluating chemicals for their potential as estrogen agonists. Estrogen antagonist activity can also be assessed with the system but HTPS methodology has not yet been extensively tested on chemicals.

3.0 Substances Used for Validation of the Proposed Test Method (See Appendix B – Characterization of substances tested)

3.1 Describe the rationale for the chemicals or products selected for use in the validation process. Include information on the suitability of chemicals selected for testing, indicating any chemicals that were found to be unsuitable.

Chemicals tested in the LUMI-CELL™ ER bioassay system included chemicals that have been reported to possess estrogenic activity as well as chemicals that have not been reported to have estrogen agonist activity. The study report conducted by the ICCVAM expert committee has established a list of 78 compounds to be tested for ER and AR transcriptional activation assays (ICCVAM 2002). In this screening mode, data could be generated in the system for validation of both positive and negative results in the LUMI-CELL™ ER bioassay system for identifying estrogenic chemicals. In analyzing a wide variety of chemicals it may also be established that the LUMI-CELL™ ER bioassay system has the potential of identifying novel estrogenic compounds or mixtures.

3.2 Discuss the rationale for the number of chemicals that were tested.

One hundred and five chemicals were tested in the LUMI-CELL™ ER BG1Luc4E2 bioassay system for this submission. 54 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 54 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay. Out of the 105 chemicals tested by LUMI-CELL™ ER bioassay system, 69 demonstrated estrogenic activity, while 36 showed no activity. Of the 51 chemicals tested, which were not included in the ICCVAM requirements for validation, 31 were found to possess' estrogenic activity, while 20 showed no activity.

3.3 Describe the chemicals/products evaluated. For each chemical or product, including the following information:

3.3.1 Chemical or product name, if a mixture, provide information on all components;

See Appendix B – Characterization of chemicals tested.

The mixtures of chemicals that were tested included 7 Arochlors, a series of chemicals, which are defined mixtures of polychlorinated biphenyls with different degrees of chlorination of the isomers.

3.3.2 CASRN

See Appendix B – Characterization of chemicals tested.

3.3.3 Chemical and product classes;

See Appendix B – Characterization of chemicals tested.

3.3.4 Physical/chemical characteristics (e.g., water and lipid solubility, pH, pKa, etc.). Any characteristics thought or know to impact the test method accuracy and/or reliability should be clearly described.

See Appendix B – Characterization of chemicals tested.

3.3.5 Stability of test substance in test medium.

See Appendix B – Characterization of chemicals tested.

Most of these chemicals are pesticides or complex polyaromatic hydrocarbons.

3.3.6 Concentrations tested;

The concentrations tested were a 10 fold dilution series of six different concentrations beginning at 10 μg/ml down to 10 pg/ml for range finding. The compounds were then re-examined in the regions which demonstrated an agonist or antagonistic response using 2 fold dilutions until a sigmoidal dose response curve was detected. Some of the positive chemicals (Diethylstilbesterol, Zearalenone, Coumesterol, Genestein, Bisphenol A, Estrone, Ethelene Estradiol, Feneramol, Kaemoferol, Methoxychlor, Norethredrel, and Diadzein) were tested more thoroughly to develop dose response characteristics and relative potency determinations.

3.3.7 Purity, including the presence and identity of contaminants and stabilizing additives;

See **Appendix B**

All of the chemicals are greater than 95% pure and generally were greater than 99% pure.

3.3.8 Supplier/source.

The suppliers for chemicals are listed below. The majority of chemicals were purchase either from the Aldrich Chemical Co., P.O. Box 355 Milwaukee, WI and Sigma Chemical Corporation, P.O. Box 14508, St. Louis, MO 68178. Some of the chemicals were purchase from Chem Service Inc., 660 Tower Lane, P.O. Box 599, West Chester, PA 19381-0599

3.4 Describe the coding procedures used in the validation studies.

Independent validation studies have not been conducted yet, and therefore coding procedures have not been used. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix H**).

3.5 For the methods that are mechanistically and functionally similar to a validated test method with established performance standards, discuss the extent to which the recommended reference chemicals were tested in the proposed test method. In situations where a listed reference chemical was unavailable, the criteria used to select a replacement chemical should be described. To the extent possible, when compared to the original reference chemical, the replacement chemical should be from the same chemical/product class and produce similar effects in the *in vivo* reference test method. In addition, if applicable, the replacement chemical should have been tested in the mechanistically and functionally similar validated test method. If applicable, the rationale for adding additional chemicals and the adequacy of data from the *in vivo* reverence test method or the species of interest should be provided.

XDS Inc. is not aware of any validated test method for detection of estrogenic endocrine disruptors. The reference compound used in this test method was 17b-estradiol (see **Appendix C**, an example of the 17b-estradiol curve and **Appendix D**, data summary). The only known direct comparison of the LUMI-CELL™ ER bioassay, system to known animal studies, was done by Jefferson et al. (2002). Please refer to section **1.1.1** (or **1.2.3**) and the attached paper in **Appendix H**.

4.0 *In Vivo* Reference Data Used for an Assessment of the Accuracy of the Proposed Test Method

The lack of reference data to establish guidelines for assessing what data constitutes information on the potential of a chemical to act as an endocrine disruptor is one of the most difficult areas to overcome in this field of research. We feel that the data provided by our LUMI-CELL™ ER bioassay system could be used as reference data to evaluate other systems for the estrogenic activity of chemicals. The system provides a rapid HTPS to evaluate and scale the potential estrogenic activity of chemicals and is based on the molecular mechanism of action of estrogenic chemicals. One method that has been suggested as a reference method for estrogenic activity is the mouse uterotrophic assay. We have initiated studies with Dr. Julius Thigpen of the National Institute of Environmental Health Sciences to compare the data generated by our LUMI-CELL™ ER bioassay system and the mouse uterotrophic assay in extracts of feed samples but that data is coded at this time and can not be presented in this filing of information to ICCVAM at this time.

The only known direct comparison of the LUMI-CELL™ ER bioassay system to known animal studies, was done by Jefferson et al. (2002). Please refer to section **1.1.2** (or **1.2.3**) and the attached paper in **Appendix H**.

4.1 Provide a clear description of the protocol(s)used to generate data from the *in vivo* reference test method. If a specific guideline has been followed, it should be provided. Any deviations should be indicated, including the rationale for the deviation.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix H**).

4.2 Provide the *in vivo* reference test method data used to assess the accuracy of the proposed test method. Individual human and/or animal reference test data, if available, should be provided. Provide the source of the reference data, including the literature citation for published data, or the laboratory study director and year generated for unpublished data.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The main sources of data used to determine accuracy of the test method have been through individual published reports and the list of compounds reposted by ICCVAM with historical data on estrogenic response (ICCVAM 2002). However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix H**).

4.3 If not included in the submission, indicate if original records are available for the *in vivo* reference test method data.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, and **1.2.3** and attached papers in **Appendix H**).

4.4 Indicate the quality of the *in vivo* reference test method data, including the extent of GLP compliance and any use of coded chemicals.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. No coded compounds have been tested as of yet. GLP guidelines were followed in the production of the LUMI-CELL™ ER bioassay. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, and **1.2.3** and attached papers in **Appendix H**).

4.5 Discuss the availability and use of relevant toxicity information from the species of interest (e.g., human studies and reported toxicity from accidental or occupational exposure for human health-related toxicity testing).

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay.

4.6 Discuss what is known or not known about the accuracy and reliability of the *in vivo* reference test method.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (please see attached paper in **Appendix H**).

5.0 Test Method Data and Results

5.1 Describe the proposed test method protocol used to generate each submitted set of data. Any differences from the proposed test method protocol should be described, and a rationale or explanation for the difference provided. Any protocol modifications made during the development process and their impact should be clearly stated for each data set.

Methods for HTPS of chemicals by our LUMI-CELL™ ER bioassay were performed as described in section 2 and Appendix A, defining the method for HTPS we are currently using. The data presented in **Appendix D, E, F** and **G** data summaries, used the same protocol described in **Appendix A**. Briefly, the cells were grown in DMEM for 4 days prior to plating. These plates were then incubated 24 hours prior to dosing with the desired compound.

5.2 Provide all data obtained using the proposed test method. This should include copies of original data from individual animals and/or individual samples, as well as derived data. The laboratory's summary judgment as to the outcome of each test should be indicated. The submission should include data (and explanations) from all studies successful or not.

In this submission for ICCVAM review we are submitting data summaries for the HTPS of the chemicals we have evaluated in the LUMI-CELL™ ER bioassay for estrogen activity. Test data summaries for each chemical screened are represented by dose response curves of the screened chemicals depicting activation of LUMI-CELL™ ER bioassay to express luciferase activity and are attached in **Appendix A**, the detailed description of the performance of the LUMI-CELL™ ER bioassay; **Appendix C**, an example of the 17b-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix E**, Antagonist Plate-to-Plate data summary; **Appendix F**, Agonist Well-to-Well data summary; and **Appendix G**, QC performance charts. All raw data files are appended to a final submission of our HTPS LUMI-CELL™ ER bioassay for estrogen activity as Excel files. The Data Summary Appendix has its corresponding Raw Data Appendix, and they are:

"**Appendix D – Plate-to-Plate Agonist Data Summary**" is a data summary for the raw data file "**Appendix D – Plate-to-Plate Agonist Raw Data**";

"**Appendix E – Plate-to-Plate Antagonist Data Summary**" is a data summary for the raw data file "**Appendix E – Plate-to-Plate Antagonist Raw Data**"

"**Appendix F – Well-to-Well Agonist Data Summary**" is the data summary for the raw data file "**Appendix F – Well-to-Well Agonist Raw Data**"

5.3 Describe the statistical approach used to evaluate the data resulting from the studies conducted with the proposed test method.

The statistical approach for evaluating data was described in section **2.2.12**.

5.4 Provide a summary, in graphic or tabular form, of the results. The suggested tabular format for providing data for use in assessment of accuracy is provided in Appendix B.

See **Appendix B** (Characterization of Substances Tested) of this report.

A useful bioassay should provide a quantitative estimate of the relative estrogenic potency of a chemical or chemical mixture. Accordingly, we reanalyzed all active compounds in our LUMI-CELL™ ER system to derive EC50 values of their activity. One hundred and five chemicals were tested in the LUMI-CELL™ ER BG1Luc4E2 bioassay system for this submission. 53 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 53 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay (ICCVAM 2002). Out of the 105 chemicals tested by LUMI-CELL™ ER bioassay system, 69 demonstrated estrogenic activity, while 41 showed no activity. Of the 51 chemicals tested, which were not included in the ICCVAM requirements for validation, 30 were found to possess' estrogenic activity, while 21 showed no activity. (see **Appendix D**, data summary)

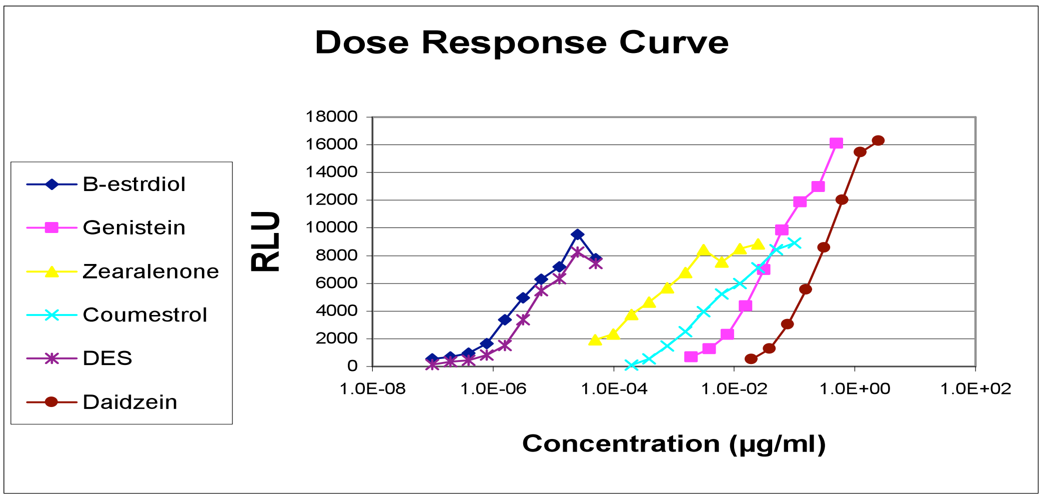

Figure 7. Repeat tests with dose response curves to evaluate EC50 response for generation of relative potency

Typical dose response characteristics of the LUMI-CELL™ ER analysis are show in Figure 7. These experiments reveal that each chemical could induce luciferase activity in BG1Luc4E2 cells in a dose-dependent manner and the differences in relative potency of a given chemical is determined by comparison of its dose-response curve to that obtained using 17ß-estradiol. Comparison of the EC_{50} values for each curve (concentration of chemical that induces luciferase to 50% of maximal) allows estimation of the relative potency of a test chemical relative to that of 17ß-estradiol. The induction potency estimates calculated from this comparison are presented in Appendix D. These results reveal that the relative potency values for these selected EDCs range from 1.5 (for DES) to about 44,000-times (for Diadzein) lower than that of 17ß-estradiol. These results demonstrate the utility of our bioassay for estimating the relative potency of other estrogenic chemicals (*i.e*. xenoestrogens).

5.5 For each set of data, indicate whether coded chemicals were tested, whether experiments were conducted without knowledge of the chemicals being tested, and the extent to which experiments followed GLP guidelines.

The laboratory is run using standard operating procedures and follows Good Laboratory practices in producing data and analytical systems. The laboratory has been audited by the Belgium Government for compliance to Good Laboratory practices since the Scientific Institute of Public Health of Belgium purchased XDS bio-analytical system for analysis of dioxin and dioxin-like chemicals. XDS is open to GLP audit by any of the US regulatory agencies and would welcome an audit and accreditation. The current experiments were not conducted in a blind coded manner. XDS has a current study being conducted on the LUMI-CELL™ ER system in collaboration with Dr. Julius Thigpen of the National Institute of Environmental Health Sciences on measuring and comparing the estrogenic activity of feed extracts. XDS would welcome double blind evaluation of the LUMI-CELL™ ER HTPS if any of the regulatory agencies would be interested in testing the system.

5.6 Indicate the "lot-to-lot" consistency of the test substances, the time frame of the various studies, and the laboratory in which the study or studies were done. A coded designation for each laboratory is acceptable.

Lot to lot consistency is conducted by comparison to the positive and negative QCs described in sections **2.2.7** and **2.4** (also see data in **Appendix G**, the QC summary charts). Inter-laboratory variability of the analysis system is currently being undertaken with the laboratory of Dr. Leo Goeyens of the Belgium Scientific Institute of Public Health and with Dr. Fujio Kayama of the Jichi Medical School of Japan and Mr. Yamamoto of the Hiyoshi Corporation of Japan. Studies have not been completed at this date.

5.7 Indicate the availability of any data not submitted for external audit, if requested.

All data analyzed at the XDS laboratory are available for audit. The current work was funded by a Phase I SBIR and Phase II SBIR grant from the National Institutes of Environmental Health Sciences, (Grant Number 1 R43 ES10533-01) "Cell Bioassays to Detect Endocrine Disruptors" and (Grant Number ES10533-03) "Recombinant Bioassays to Detect Endocrine Disruptors". We appreciate the funding supplied by NIEHS and support of the Dr. Jerry Heindel in aiding development of the LUMI-CELL® ER bioassay.

6.0 Test Method Accuracy

Test method performance for estrogen active chemicals is difficult in that there is not an accepted and validated test procedure that we are aware of. We are pursuing ICCVAM submission of our data to begin the process of having our analysis system evaluated for performance and hope to work with ICCVAM and the other regulatory agencies to accomplish this goal.

6.1 Describe the accuracy (e.g., concordance, sensitivity, specificity, positive and negative predictivity, false positive and negative rates) of the proposed test method compared with the reference test method. Explain how discordant results in the same or multiple laboratories from the proposed test were considered when calculating accuracy.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. Sense there is no validated test method false positive and false negative rates can not be established. But there is considerable consistency between the LUMI-CELL™ ER bioassay and the data published in the Jefferson et. al. (2002) paper. More specifically, the LUMI-CELL™ ER bioassay and the Gland number assay showed 100% consistency. While the other assays (uterotropic assay, Cell height assay and LF assay all showed false negatives in that they did not demonstrate activity for all of the compounds tested (Jefferson, Padilla-Banks et al. 2002) (see attached paper in **Appendix H**).

6.2 Discuss results that are discordant with results from the *in vivo* reference method.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. Nine of the compounds tested by Jefferson et. al. (2002) were also tested by XDS and came up with very consistent results. When Jefferson et. al. (2002) compared the LUMI-CELL™ ER bioassay to the Gland number assay they showed 100% consistency. The Uterotrophic assay was able to detect activity in 7 of 9 compounds tested, and was not able to detect Daidzein nor Naringenin. The Cell height assay was able to detect activity in 8 of 9 compounds tested, and was not able to detect Daidzein. The LF protein assay was able to detect activity in 7 of 9 compounds tested, and was not able to detect activity in Biochanin A nor Daizedin. (see attached paper in **Appendix H**).

6.3 Discuss the accuracy of the proposed test method compared to data or recognized toxicity from the species of interest (e.g., humans for human health-related toxicity testing), where such data or toxicity classifications are available. This is essential when the method is measuring or predicting an endpoint for which there is no preexisting method. In instances where the proposed test method was discordant reference test method, describe the frequency of correct predictions of each test method compared to recognized toxicity information from the species of interest.

Most of the historical data on compound response has come from the ICCVAM publication "Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation" (ICCVAM 2002). One hundred and five chemicals were tested in the LUMI-CELL™ ER bioassay system for this submission. 53 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 53 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay. Out of the 105 chemicals tested by LUMI-CELL™ ER bioassay system, 69 demonstrated estrogenic activity, while 35 showed no activity. Of the 51 chemicals tested, which were not included in the ICCVAM requirements for validation, 30 were found to possess' estrogenic activity, while 21 showed no activity (see **Appendix D**, data summary).

6.4 State the strengths and limitations of the method, including those applicable to specific chemical classes or physical-chemical properties.

The strengths of the LUMI-CELL™ ER system have been highlighted in previous sections (particularly introduction and sections **1.2.1, 1.2.2, 1.2.3**, and **1.2.4**). Briefly, one of strengths of the LUMI-CELL™ ER system is that it is a mechanistically based bioassay system that measure function of the estrogen receptor system and the effects of chemicals on this system. The assay is rapid, economical, and provides relative potency of chemicals due to the large dynamic range of the system as demonstrated in the data in section **5** (particularly section **5.4** and **Appendix C**, an example of the 17b-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix E**, Antagonist Plate-to-Plate data summary; **Appendix F**, Agonist Well-to-Well data summary; and **Appendix G**, QC performance charts). The luciferase endpoint is easy to measure (production of light) and quantify and it is specific since this gene is not normal to the cell making background expression controllable. The only limitation of the method is that it requires the cells to be alive to respond and can not test acutely toxic chemicals, which are toxic at concentration thought to be active.

6.5 Describe the salient issues of data interpretation, including why specific parameters were selected for inclusion.

Parameters such as inclusion of 8 QC points in addition to the 11 point, 17b-estradiol dose response curve were included to preserve the integrity of the system (see **Appendix C**, an example of the 17b-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; and **Appendix G**, QC performance charts).

6.6 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results obtained with both test methods should be compared with each other and with the *in vivo* reference test method and/or toxicity information from the species of interest.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, **1.2.3** and **6.2** and attached papers in **Appendix H**).

7.0 Test Method Reliability (Repeatability/Reproducibility)

7.1 Discuss the selection rationale for the substances used to evaluate the reliability (intra-laboratory repeatability and intra- and inter-laboratory reproducibility) of the proposed test method as well as the extent to which the chosen set of chemicals represents the range of possible test outcomes.

The basis for test chemical selection was in 2 parts, those recommended by ICCVAM (ICCVAM 2002) and other chemicals which were selected since they have been reported as potential estrogen active chemicals. Most of the other chemicals are known environmental contaminants and evaluation of their potential as estrogen agonists is needed. Many compounds with historical data for positive and negative for both agonistic and antagonistic response were used in theses studies. Most of the historical data on these compounds came from the ICCVAM publication “Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation” (ICCVAM 2002).

7.2 Provide analyses and conclusions reached regarding inter- and intra-laboratory repeatability and reproducibility. Acceptable methods of analyses include those described in ASTM E691-92 (6) or by coefficient of variation analysis.

Coefficient of variation analysis has been conducted for both the agonist plate-to-plate and well-to-well variability as well as the antagonist variability of LUMI-CELL™ ER bioassay. This data is available in **Appendix C**, an example of the 17b-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix E**, Antagonist Plate-to-Plate data summary; **Appendix F**, Agonist Well-to-Well data summary; and **Appendix G**, QC performance charts.

Intra-laboratory validation has not been done at this time but studies are underway with the investigators mentioned in section 5.5 Dr. Goeyens of the Scientific Institute of Public Health of Belgium and Dr. Kayama of Jichi Medical School and Mr. Yamamoto of the Hiyoshi Corporation of Japan.

Also the only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2**, **1.2.3** and **6.2** and attached papers in **Appendix H**).

7.3 Summarize historical positive and negative control data, including number of experiments, measures of central tendency, and variability.

The only historical data available on the substances used comes from the ICCVAM report (ICCVAM 2002) and other individual papers describing specific compounds. We would like to establish the LUMI-CELL™ ER bioassay as a quick, reliable method by which to produce historical data in regard to putative estrogenic endocrine disruptors. The dose response curves should be able to refine and reduce the use of animals by giving a more clear understanding of the active ranges of suspect compounds.

7.4 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the reliability of the two test methods should be compared and any differences discussed.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

8.0 Test Method Data Quality

8.1 State the extent of adherence to national and international GLP guidelines (7-12) for all submitted data, including that for the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method. Information regarding the use of coded chemicals and coded testing should be included.

The laboratory as discussed above follows GLP guidelines and audit of the laboratory for GLP compliance has been done by the Belgium Government. Coded studies are underway but not yet completed. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

8.2 Summarize the results of any data quality audits, if conducted.

No data quality audits have been conducted to this point.

8.3 Discuss the impact of deviations from GLP guidelines or any noncompliance detected in the data quality audits.

No deviations from the GLP guidelines have occurred, and no data quality audits have been conducted to this point.

8.4 Address the availability of laboratory notebooks or other records for an independent audit. Unpublished data should be supported by laboratory notebooks.

All records and notebooks are available for viewing upon request from independent auditors.

9.0 Other Scientific Reports and Reviews

9.1 Summarize all available and relevant data from other published or unpublished studies conducted using the proposed test method.

Appended is a peer reviewed scientific publication by Rogers and Denison on the BG1Luc4E2 system (Rogers and Denison 2000) and a published paper for the Dioxins 2003 conference (Gordon, Chu et al. 2003), 2 abstracts, one submitted to SOT 2004 (Gordon, Chu et al. 2004) the other to e.hormone 2003

(Gordon, Chu et al. 2003)and a paper currently being reviewed for submission (Gordon, Chu et al. 2004). The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

9.2 Comment on and compare the conclusions published in independent peer- reviewed reports or other independent scientific reviews of the proposed test method. The conclusions of such scientific reports and reviews should be compared to the conclusions reached in this submission. Any ongoing evaluations of the proposed test method should be described.

The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

9.3 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results of studies conducted with the validated test method subsequent to the ICCVAM evaluation should be included and any impact on the reliability and accuracy of the proposed test method should be discussed.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement)

10.1 Describe how the proposed test method will refine (reduce or eliminate pain or distress), reduce, and/or replace animal use compared to the current methods used.

The LUMI-CELL™ ER bioassay is an *in vitro* system and could be used to reduce, refine and replace the number of animal tests now being conducted, if it is validated and found to be predictive of estrogen agonists and antagonists. The system is at the early stages of validation and need to be further explored on how well it could replace animal systems.

10.2 If the proposed test method requires the use of animals, the following items should be addressed:

10.2.1 Describe the rationale for the need to use animals and describe why the information provided by the proposed test method requires the use of animals (i.e., cannot be obtained using non-animal methods).

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

10.2.2 Include a description of the sources used to determine the availability of alternative test methods that might further refine, reduce, or replace animal use for this testing. This should, at a minimum, include the databases searched, the search strategy used, the search date(s), a discussion of the results of the search, and the rationale for not incorporating available alternative methods.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay, therefore would be used to refine, reduce and replace animals used in testing. Since there is no other validated test for the detection of estrogenic endocrine disruptors, this method would fit these requirements completely. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

10.2.3 Describe the basis for determining that the number of animals used is appropriate.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

10.2.4 If the proposed test method involves potential animal pain and distress, discuss the methods and approaches that have been incorporated to minimize and, whenever possible, eliminate the occurrence of such pain and distress.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

11.0 Practical Considerations

11.1 Discuss the following aspects of test method transferability. Include an explanation of how this compares to the transferability of the reference test method and, if applicable, to a comparable validated test method with established performance standards.

XDS is unaware of an accepted reference test method. One of the many methods suggested is the mouse uterotrophic assay that requires specialized animal facilities, large numbers of animals, and highly trained individuals to evaluate results. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**).

11.1.1 Discuss the facilities and major fixed equipment needed to conduct a study using the test method.

The equipment and supplies need to perform the test are detailed in section 2. The facilities required are a functioning laboratory. Our current facility is a 1600 square foot laboratory but all analysis can be performed in single room (i.e. 10 foot by 20-foot tissue culture facility).

11.1.2 Discuss the general availability of other necessary equipment and supplies.

All equipment necessary for the LUMI-CELL™ ER bioassay is readily available from the suppliers listed in section **2**.

11.2 Discuss the following aspects of proposed test method training. Include an explanation of how this compares to the level of training required to conduct the *in vivo* reference test method and, if applicable, a comparable validated test method with established performance standards.

11.2.1 Discuss the required level of training and expertise needed for personnel to conduct the proposed test method

There is a certain level of training needed to conduct the proposed test method. But, this training can easily be conducted by XDS staff. For cross lab validation the training would be minimal if the four labs currently using XDS's CALUX® method are used.

11.2.2 Indicate any training requirements needed for personnel to demonstrate proficiency and describe any laboratory proficiency criteria that should be met.

Persons should be adept at cell culture and organic extractions.

11.3 Cost Considerations - Discuss the cost involved in conducting a study with the proposed test method. Discuss how this compares to the cost of the *in vivo* reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However the cost considerations are limited to the equipment and supplies listed in section **2**.

11.4 Time Considerations - Indicate the amount of time needed to conduct a study using the proposed test method and discuss how this compares with the *in vivo* reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

Once the cell line is established and growing (see **Appendix A**, detailed description of performance of the LUMI-CELL™ ER bioassay), studies can be conducted in as little as 48 hours. There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However, current in vivo studies take anywhere from several weeks, with the uterotrophic assays, to years, with the 2 gen studies, to conduct.

12.0 References cited:

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13.0 Supporting Materials

13.1 Provide the complete, detailed protocol for the proposed test method.

See **Appendix A** - detailed description of performance of the LUMI-CELL™ ER bioassay

13.2 Provide the detailed protocol(s) used to generate reference data for this submission and any protocols used to generate validation data that differ from the proposed protocol.

There was no data generated which differs from the protocol in **Appendix A**, detailed description of performance of the LUMI-CELL™ ER bioassay.

13.3 Provide copies of all relevant publications, including those containing data from the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method with established performance standards.

The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix H**). Copies of all publications are attached here as appendices.

13.4 Include all available non-transformed original data for both the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method with established performance standards.

See **Appendix C**, an example of the 17b-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix E**, Antagonist Plate-to-Plate data summary; **Appendix F**, Agonist Well-to-Well data summary; and **Appendix G**, QC performance charts.

13.5 If appropriate performance standards for the proposed test method do not exist, performance standards for consideration by NICEATM and ICCVAM may be proposed. Examples of established performance standards can be located on the ICCVAM/NICEATM web site at http://iccvam.niehs.nih.gov.

Performance standards are available from ICCVAM (ICCVAM 2002)and were used in this study. QC performance charts can be found in **Appendix G**.

Appendix A: Detailed Description of Performance of the LUMI-CELL™ ER bioassay

Mass culture of BG1Luc4E2 cell line: The cell line BG1Luc4E2 has remained stably transfected with the reporter plasmid for over 5 years. Early clones of the cells are stored in liquid nitrogen in 1 ml ampoules. Mass culture of the cells are initiated by quickly thawing an ampoule and suspending in 5 ml of RPMI 1640 containing 5% fetal calf serum (Hyclone) and 1% pen/strep solution (RPMI growth media) in a T25 culture flask. The cells are then incubated in a 5% CO2 incubator at 37C and allowed to grow to confluence (approximately 24 hrs). The adherent BG1Luc4E2 cells are rinsed with Phosphate Buffered Saline (PBS) and a 1% solution of Trypsin (Gibco) is applied to the cells in the flask and they are incubated in this solution for approximately 5 minutes. Hit the side of flask sharply against heel of your palm to dislodge cells from the bottom of the flask and confirm release of the cells by visual examination with an inverted microscope. If not, allow to incubate for another 30 seconds and try again. If cells have been mostly dislodged, then add 5-6 milliliters PBS to the flask and wash the cells out of the flask. The cells are washed out of the flask with PBS and are transferred to a 50 ml centrifuge tube. RPMI 1640 Media containing 5% fetal calf serum is immediately added to the tube to inhibit further cellular digestion by residual Trypsin. The cells are pelleted by centrifugation in a desk top clinical centrifuge at 1000 RPM and resuspended in 2 ml of RPMI growth media and repeatedly drawn through a pipette to break up clumps of cells. One ml of the pelleted cells is transferred inoculated into two T75 flask containing 10 ml of RPMI growth media and allowed to grow in the incubator at 37 C with 5% CO2 atmosphere until confluent.

The cells from one T75 flask can be used to inoculate four T75 flasks by repeating the trypsin procedure for passage of cells described above.

Conditioning of BG1Luc4E2 cells for measuring estrogen dependent luciferase activity:
The BG1Luc4E2 cells must be grown in estrogen free conditions to allow measurement of estrogen dependent induction of luciferase activity [Rogers, 2000 #197]. The following procedure has been developed to condition the cells in estrogen free conditions and then allow plating of the cells in a 96 well plate format for HTPS analysis of estrogen dependent induction of luciferase activity. Two T75 flasks of cells grown in RPMI growth media that have reached confluence are removed from the incubator and the media is poured off of the cells. The cells are washed with 5 ml of PBS and a 1% solution of Trypsin (Gibco) is applied to the cells in the flask and they are incubated in this solution for approximately 5 minutes. Hit the side of flask sharply against heel of your palm to dislodge cells from the bottom of the flask and confirm release of the cells by visual examination with an inverted microscope. If not, allow to incubate for another 30 seconds and try again. If cells have been mostly dislodged, then add 5-6 milliliters PBS to the flask and wash the cells out of the flask. The cells are washed out of the flask with PBS and are transferred to a 50 ml centrifuge tube. DMEM Media containing 5% fetal calf serum (that has been stripped of estrogen by treatment with activated carbon and is free of phenol red pH indicator) is immediately added to the tube to inhibit further cellular digestion by residual Trypsin. The cells are pelleted by centrifugation in a desk top clinical centrifuge at 1000 RPM and resuspended in 3.5 ml of DMEM growth media and repeatedly drawn through a pipette to break up clumps of cells. Seven hundred ml of the pelleted cells is transferred inoculated into two T75 flask containing 10 ml of RPMI growth media and 2 flasks containing 10 ml DMEM and allowed to grow in the incubator at 37^{o} C with 5% CO2 atmosphere until confluent.. Estrogen free media consists of Dulbeco's Minimal Essential Media supplemented with 5% fetal calf serum that has been stripped of estrogen by treatment with activated carbon and is free of phenol red pH indicator. The flasks of cells are returned to the incubator for 4 days. At this time the cells are ready to be plated into 96 well plates. The two T75 flasks are treated with trypsin and cells washed with PBS and the cells are resuspended in approximately 15 ml of Estrogen free media. The cells are counted with a hemocytometer and adjusted to a concentration of 200,000 cells per ml in Estrogen free media. Two hundred microliters of media are dispensed into each well of a 96 well plate (60,000 cells per well). The plate is returned to the incubator of 24 hours to allow them to adhere and grow in the plate.

Dosing 96 well plates of BG1Luc4E2 cells with Estrogen and test compounds:
To determine the agonistic response, dilutions of 17b-estradiol and test compounds are prepared in DMSO. A standard solution of 10 ng/ml of 17b-estradiol in DMSO is used to prepare dilutions of this standard. Four microliters of DMSO is added to ten 13 mm glass tubes. Four microliters of the 10 ng/ml standard solution of 17b-estradiol is added to both the first tube (not containing DMSO) and the second tube containing the 4 microliters of DMSO in the tube. The second tube is vortexed and four microliters transferred to the next tube in the series. This is repeated for each of the 10 tubes creating a two fold dilution series. For the antagonistic response, A standard solution of 5 µg/ml of Tamoxifen in DMSO is used to prepare dilutions of this standard. Four microliters of DMSO is added to ten 13 mm glass tubes. To the first tube 4 microliters of the 5 µg/ml standard solution of Tamoxifen is added to both the first tube (not containing DMSO) and second tube containing the 4 microliters of DMSO in the tube. The second tube is vortexed and four microliters transferred to the next tube in the series. This is repeated for each of the 10 tubes creating a two fold dilution series. Then 4 µl of a 5 ng/ml 17b-estradiol standard solution is added to each tube, vortexed and 4 µl removed from each tube to keep the total volume of DMSO at 4 µl. Four microliters of all appropriate positive and negative control QCs are also added to separate tubes to ensure the integrity of the system. For this system that would include 4 DMSO QCs, 1 no DMSO QC (i.e. just media) and 3 positive response QCs (Bisphenol A, Estrone, and DES). Also, if appropriate, the cell viability assay was used. The cell viability assay includes adding 4 µl of 5 ng/ml or 0.5 ng/ml 17b-estradiol standard solution to the highest concentration of the test substance being analyzed (and to ½ and $1/10^{th}$ the highest concentration tested if antagonism is suspected). To each tube 400 microliters of Estrogen free media is added to the DMSO solution and the tube vortexed vigorously. Similar dilution series are produced for test compounds or extracts being analyzed for estrogenic activity by the BG1Luc4E2 cells.

The 96 well plates of cells are removed from the incubator and media removed from the adherent cells by inversion onto absorbent plastic backed paper. The cells are rinsed with 50 microliters of PBS and this also removed by inversion on absorbent plastic backed paper. Two hundred microliters of beta-estradiol solutions or test compound is then applied to the 96 well plates. The outside rows of the plate are not used for determinations since we have found that these wells are very sensitive to environmental conditions and do not provide reproducible quantitative readings for induction of luciferase activity with the BG1Luc4E2 cells. The dosed plates of BG1Luc4E2 are returned to the incubator and incubated for 24 hours to allow maximal induction of luciferase activity in the cells.

<u>Measurement of estrogen induced luciferase activity in BG1Luc4E2 cells:</u>
Luciferase that is produced in the BG1Luc4E2 cells in response to exposure to estrogen accumulates in the cytoplasm of the cells over the twenty-four hour incubation. To measure luciferase the cells must be lysed and substrates for measurement of luciferase enzyme activity added and results, light emission by the enzymatic activity measured in a luminometer. To accomplish this, the cells are removed from the incubator and media removed by inversion of the plate on absorbent plastic backed paper, and the plates tapped on the paper to remove residual media. The cells in the 96 well plates are washed with PBS and the cells examined with an inverted microscope to observe whether any observable toxicity or displacement of the lawn of cells grown on the bottom of the plates has occurred. The PBS is then removed by inversion of the plate on absorbent plastic backed paper and a reflective white plate tape (Packard) applied to the clear bottoms of the 96 well plates to increase the efficiency in measuring emitted light from the wells of the plate. A dilute detergent lysis reagent (Promega) is then added to the cells and the cells shaken in a vibrating mixer to aid in lysis of the cells. The cells are then placed in a Berthold Orion Microplate Luminometer which is a robotic instrument that delivers 50 microliters of luciferase enzyme reagent (Promega) to each well and then measures the resulting light omitted (integrating light emission from the wells for a 15 second period). The light emission is expressed as Relative Light Units (RLU) for each well. The measured RLU by the instrument is then exported to a Compaq computer and analyzed with software designed to provide analysis of the RLU of the 17b-estradiol standard, subtraction of blank responses and interpolation of unknown responses to the standard curve.

Shown in figures 3 through 6 are sample outputs of a typical HTPS data analysis system XDS has developed for estimation of the estrogenic activity of chemicals and extracts of environmental samples. A sample template for the 96 well plate analysis is shown in Figure 3 and it includes, the 17b-estradiol standard, test chemicals for analysis of potential estrogenic activity, and measurements of extracts of environmental chemicals for luciferase activity as well as control samples of background or solvent blank in the system. As described earlier, we have determined that the responsiveness of the BG1Luc4E2 cells is sensitive to an edge effect in which determinations made in the outer wells of the plate are variable and result in reduced confidence of analysis of luciferase activity in these wells. Therefore, on a 96 well plate 56 wells of the plate are useful for determination of estrogen dependent induction of luciferase activity. The ten standard dilutions of 17b-estradiol are therefore applied to wells B2 through E3. This provides a standard curve of 17b-estradiol in a two fold dilution series from 50 pg/ml down to 0.097 pg/ml. Solvent or blank controls are applied to a number of wells of the plate to provide replicate of estimates of the background response expected for luciferase expression by the BG1Luc4E2 cells (wells F3, G3, B4, F10, G10). In this example plate, sample extracts and replicate extractions of these extracts have been applied to wells C4 through E10. A dilution series of the chemical diethylstilbesterol that is a known estrogen active agonist has been applied to wells B11 through G11 of the plate.

	1	2	3	4	5	6	7	8	9 20 hour exposure	10	11 extrogen cells	12
A												
B		5.00E+01 B-estradiol	7.81E-01 B-estrdiol	DMSO blank	19-6 A01341 1000	19-12 A01343 1000	19-18 A00844 1000	19-24 A00849 1000	19-30 A00918 1000	19-36 A00993 1000	5.00E+01 DES	
C		2.50E+01 B-estrdiol	3.91E-01 B-estrdiol	19-1 A01340 1000	19-7 A01342 1000	19-13 A00843 1000	19-19 A00848 1000	19-25 A00894 1000	19-31 A00919 1000	19-37 A01007 1000	2.50E+01 DES	
D		1.25E+01 B-estrdiol	1.95E-01 B-estrdiol	19-2 A01340 1000	19-8 A01342 1000	19-14 A00843 1000	19-20 A00848 1000	19-26 A00894 1000	19-32 A00919 1000	19-38 A01007 1000	1.25E+01 DES	
E		6.25E+00 B-estrdiol	9.77E-02 B-estrdiol	19-3 A01340 1000	19-9 A01342 1000	19-15 A00843 1000	19-21 A00848 1000	19-27 A00894 1000	19-33 A00919 1000	19-39 A01007 1000	6.25E+00 DES	
F		3.13E+00 B-estrdiol	DMSO blank	19-4 A01341 1000	19-10 A01343 1000	19-16 A00844 1000	19-22 A00849 1000	19-28 A00918 1000	19-34 A00993 1000	19-40 Extraction blank 1000	3.13E+00 DES	
G		1.56E+00 B-estrdiol	DMSO blank	19-5 A01341 1000	19-11 A01343 1000	19-17 A00844 1000	19-23 A00849 1000	19-29 A00918 1000	19-35 A00993 1000	19-41 Extraction blank 1000	1.56E+00 DES	
H												

Figure 3 Template

rosys anthos Test Name: **ESTROGEN**
Lucy 1 Cell line ID: Estrogen

Microplate	PACKARD OPTIPLATE 96	No. of Intervals	50	Interval Time [s]	0.3
Layout	XDS 96 well (Inside)	Tot. Meas. Time/Well [s]	15	Start Measurement [s]	0
		Start Injection 1 [s]	0	Start Injection 2 [s]	15

Test Type Well Mode
Reading Direction vertical

Calculation Range Start 5 Stop 50

Multiple:

Raw Data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		13932	3987	2164	6401	9802	6512	7436	7825	5798	12641	
C		13019	3271	9282	8921	6051	3973	8231	5725	8303	12570	
D		11768	3067	10023	9121	5933	3793	8133	5696	9455	10680	
E		10190	2916	9372	8532	6665	4044	7782	5275	8295	9222	
F		8167	3121	6470	8906	7096	7068	6997	6101	3320	6574	
G		6314	2590	7384	9696	6150	6119	6538	5742	3298	4705	
H												

Raw Data - Blank

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		11342	1397	-426	3811	7212	3922	4846	5235	3208	10051	
C		10429	681	6692	6331	3461	1383	5641	3135	5713	9980	
D		9178	477	7433	6531	3343	1203	5543	3106	6865	8090	
E		7600	326	6782	5942	4075	1454	5192	2685	5705	6632	
F		5577	531	3880	6316	4506	4478	4407	3511	730	3984	
G		3724	0	4794	7106	3560	3529	3948	3152	708	2115	
H												

blank 2590

Figure 4 Raw Data

THE MODEL:

RLU = (v*(d^n))/(d^n + k^n)

'd' is the natural logarithm of b-Estradiol concentration
"v" is the limiting value of the RLU response as b-Estradiol concentration increases
"k" is the dose at which the response is 50% of maximum
"n" is a parameter that determines sigmoidal shape of curve
"b" is the interecept parameter

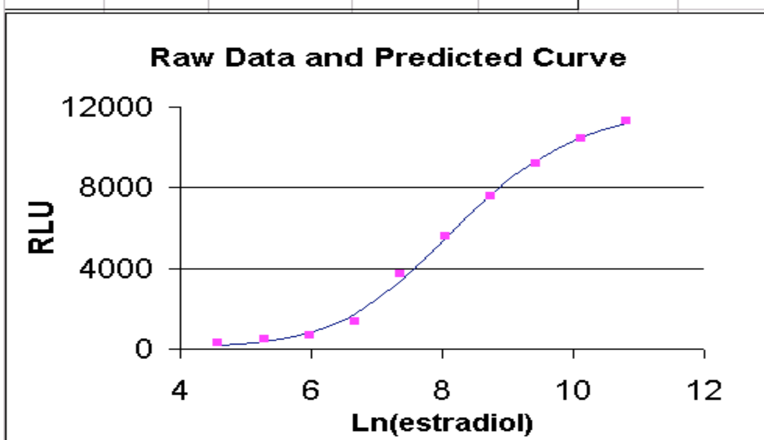
Initial Values (replaced with final estimates by 'Solver')

k	8.26314
n	8.681861
v	12138.97
b	122.946

To fit standard curve, paste b-Estradiol and RLU data into the framed columns below. Then select 'Solver; from the Tools menu. Be sure that the 'Target Cell' is set to **D43**, the 'Equal To' option is set to **min**, and the 'By Changing Cells' option is set to **B8:B11**

b-Estradiol	Ln(b-Estradiol)	RLU
50000.00	10.82	11342.20
25000.00	10.13	10428.60
12500.00	9.43	9178.40
6250.00	8.74	7599.80
3125.00	8.05	5576.60
1562.50	7.35	3724.20
781.25	6.66	1397.40
390.63	5.97	680.60
195.31	5.27	476.60
97.66	4.58	326.40

Pred	Res	Res^2
11195.71	-146.49	21459.67
10488.50	59.90	3587.80
9342.64	164.24	26975.49
7643.06	43.26	1871.73
5497.77	-78.83	6214.28
3359.27	-364.93	133172.63
1742.07	344.67	118794.37
802.34	121.74	14820.40
364.42	-112.18	12583.29
195.02	-131.38	17259.42
		3.57E+05



CONFIDENTIAL

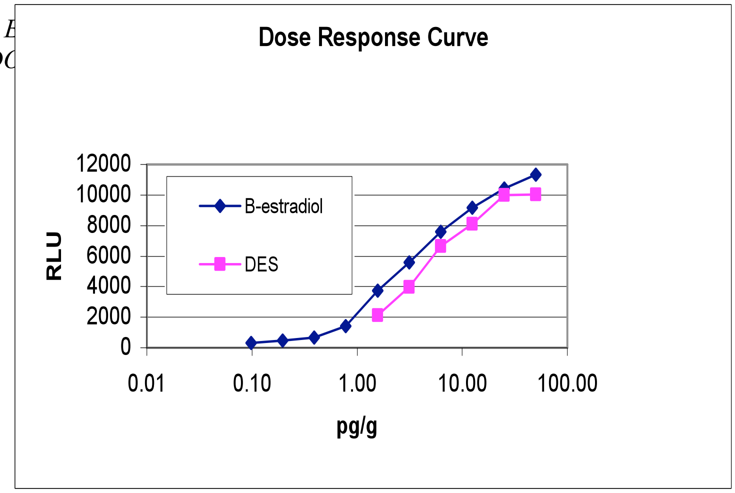
To obtain predicted Ln(b-Estradiol) concentrations from observed RLU data, paste observed RLU values into the framed cells below. These are your predicted concentrations based on the standard curve

Observed Response	Predicted Ln(b-Estradiol)	pg
6691.60	8.42154488	4.543918
7433.00	8.667346021	5.810059
6781.80	8.450660626	4.678162
3879.60	7.533498207	1.869634
4794.20	7.828456114	2.51105
3811.20	7.510536373	1.827193
6331.00	8.306720269	4.051005
6530.80	8.370056357	4.315879
5942.40	8.185050528	3.586925
6316.00	8.301990224	4.031889
7106.00	8.556954047	5.202809
7212.00	8.592350163	5.390267
3461.00	7.390081142	1.619838
3343.00	7.34822827	1.553442
4075.40	7.59837297	1.994947
4506.40	7.73755028	2.292849
3560.20	7.424738664	1.676961
3921.60	7.547517474	1.89603
1382.80	6.446182787	0.630292
1203.00	6.320913156	0.556081
1453.60	6.49178651	0.659701
4477.60	7.728377418	2.271913
3529.00	7.413887643	1.658863
4846.40	7.844818701	2.552475
5640.60	8.091402174	3.266264
5543.40	8.061311813	3.169445
5192.40	7.952605275	2.842972
4407.20	7.705887091	2.221387
3947.80	7.556233161	1.912627
5235.40	7.965941926	2.881142
3135.00	7.272589737	1.440276
3106.00	7.261836061	1.42487
2684.60	7.09867482	1.210362
3510.60	7.407467391	1.648247
3151.60	7.278721188	1.449134
3207.60	7.29927968	1.479234
5713.40	8.113953583	3.34076
6865.20	8.477746622	4.806607
5705.00	8.111350613	3.332075
729.80	5.886357422	0.360091
708.00	5.860330414	0.35084
5543.40	8.061311813	3.169445
5192.40	7.952605275	2.842972
4407.20	7.705887091	2.221387
3947.80	7.556233161	1.912627
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!
5235.40	7.965941926	2.881142
3135.00	7.272589737	1.440276
3106.00	7.261836061	1.42487
2684.60	7.09867482	1.210362
3510.60	7.407467391	1.648247
3151.60	7.278721188	1.449134
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!
3207.60	7.29927968	1.479234
5713.40	8.113953583	3.34076

Figure 5 Calculation Model

b-Estradiol

pg/ml	RLU
50.00	11342
25.00	10429
12.50	9178
6.25	7600
3.13	5577
1.56	3724
0.78	1397
0.39	681
0.20	477
0.10	326



DES

pg/ml	RLU
50.00	10051
25.00	9980
12.50	8090
6.25	6632
3.13	3984
1.56	2115

CALCULATIONS

Sample	Client ID	Identity	Fraction	RLU	TEQ, pg/ml	ppt/sample	ng/sample	-bkg	Mean	STD	%std
A01340	964-00	19-1	1000	6692	4.54	908.78	0.91	0.84	0.93	0.14	15%
A01340	964-00	19-2	1000	7433	5.81	1162.01	1.16	1.09			
A01340	964-00	19-3	1000	6782	4.68	935.63	0.94	0.86			
A01341	3327-99	19-4	1000	3880	1.87	373.93	0.37	0.30	0.34	0.08	22%
A01341	3327-99	19-5	1000	4794	2.51	502.21	0.50	0.43			
A01341	3327-99	19-6	1000	3811	1.83	365.44	0.37	0.29			
A01342	3627-00	19-7	1000	6331	4.05	810.20	0.81	0.74	0.72	0.07	10%
A01342	3627-00	19-8	1000	6531	4.32	863.18	0.86	0.79			
A01342	3627-00	19-9	1000	5942	3.59	717.38	0.72	0.65			
A01343	8708-00	19-10	1000	6316	4.03	806.38	0.81	0.73	0.90	0.15	16%
A01343	8708-00	19-11	1000	7106	5.20	1040.56	1.04	0.97			
A01343	8708-00	19-12	1000	7212	5.39	1078.05	1.08	1.01			
A00843	6637-99	19-13	1000	3461	1.62	323.97	0.32	0.25	0.27	0.05	17%
A00843	6637-99	19-14	1000	3343	1.55	310.69	0.31	0.24			
A00843	6637-99	19-15	1000	4075	1.99	398.99	0.40	0.33			
A00844	6640-99	19-16	1000	4506	2.29	458.57	0.46	0.39	0.32	0.06	20%
A00844	6640-99	19-17	1000	3560	1.68	335.39	0.34	0.26			
A00844	6640-99	19-18	1000	3922	1.90	379.21	0.38	0.31			
A00848	963-00	19-19	1000	1383	0.63	126.06	0.13	0.05	0.05	0.01	21%
A00848	963-00	19-20	1000	1203	0.56	111.22	0.11	0.04			
A00848	963-00	19-21	1000	1454	0.66	131.94	0.13	0.06			
A00849	966-00	19-22	1000	4478	2.27	454.38	0.45	0.38	0.36	0.09	25%
A00849	966-00	19-23	1000	3529	1.66	331.77	0.33	0.26			
A00849	966-00	19-24	1000	4846	2.55	510.49	0.51	0.44			
A00894	964-00	19-25	1000	5641	3.27	653.25	0.65	0.58	0.55	0.04	8%
A00894	964-00	19-26	1000	5543	3.17	633.89	0.63	0.56			
A00894	964-00	19-27	1000	5192	2.84	568.59	0.57	0.50			
A00918	966-00	19-28	1000	4407	2.22	444.28	0.44	0.37	0.40	0.10	25%
A00918	966-00	19-29	1000	3948	1.91	382.53	0.38	0.31			
A00918	966-00	19-30	1000	5235	2.88	576.23	0.58	0.50			
A00919	8537A-99	19-31	1000	3135	1.44	288.06	0.29	0.22	0.20	0.03	13%
A00919	8537A-99	19-32	1000	3106	1.42	284.97	0.28	0.21			
A00919	8537A-99	19-33	1000	2685	1.21	242.07	0.24	0.17			
A00993	1067-00	19-34	1000	3511	1.65	329.65	0.33	0.26	0.23	0.02	9%
A00993	1067-00	19-35	1000	3152	1.45	289.83	0.29	0.22			
A00993	1067-00	19-36	1000	3208	1.48	295.85	0.30	0.22			
A01007	7865-00	19-37	1000	5713	3.34	668.15	0.67	0.60	0.69	0.17	24%
A01007	7865-00	19-38	1000	6865	4.81	961.32	0.96	0.89			
A01007	7865-00	19-39	1000	5705	3.33	666.42	0.67	0.59			
Extraction	19-40	19-40	1000	730	0.36	72.02	0.07				
Extraction	19-41	19-41	1000	708	0.35	70.17	0.07				

Figure 6 Estrogen Report

Figure 4 displays a typical luminometer output from an analysis. The relative light units (RLU) measured for each well of the test plate are shown in Table 1, while Table 2 displays the RLU corrected for the selected background wells, F3 and G3. These data are then exported to a program designed by XDS for graphic display of the results and calculation of the estrogenic activity of samples based on extrapolation to the standard curve of 17b-estradiol.

We have determined that the output of receptor mediated gene expression systems is best estimated by a 4 parameter Hill equation. The Hill equation that we are using to extrapolate receptor mediated gene expression is shown at the top of Figure 5. Input of the RLU for samples is entered into this equation and the pg of estrogenic like activity for the sample is estimated from the model. The output is expressed as pg of estrogenic activity derived from the model.

Figure 6 displays the output of the analysis corrected for the amount of sample extracted for the determination. The estimated estrogenic activity of each sample from Figure 5 are corrected for the dilution of sample extract that was used in the analysis (Figure 6). This analysis also displays a non-modeled graphic display of the data. The figure provides a graphic display of the output from the 17b-estradiol standard and the test chemical diethylstilbesterol that was analyzed on this plate. The diethylstilbesterol analysis was a two-fold dilution analysis from 50 pg/ml down to a concentration of 1.56 pg/ml. The table output provides an estimate of the estrogenic activity in pg per ml (or parts per trillion of estrogenic activity in the sample; column 6 of the output) for extracts of feed samples analyzed in this analysis. Triplicate analysis of the feed extract samples were performed and mean and standard deviation for estrogenic activity of the sample is expressed as ng Estrogenic activity per gram of sample or parts per billion (ppb).